Redox Potential Controls the Structure and DNA Binding Activity of the Paired Domain*

(Received for publication, April 22, 1998, and in revised form, July 9, 1998)

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Pax proteins are transcriptional regulators controlling a variety of cell fates during animal development. This role depends on the intact function of the paired (Prd) domain that is able to recognize specific DNA sequences. The Prd domain is composed of two distinct helix-turn-helix subdomains, PAI and RED. Molecular functions of Pax proteins are subjected to different levels of regulation involving both pre-translational and post-translational mechanisms. By using Pax-5 and Pax-8 recombinant proteins, we demonstrate that the binding activity of the Prd domain is regulated through the oxidation/reduction of conserved cysteine residues. Mass spectrometry analysis and mutagenesis experiments demonstrate that the redox regulation is accomplished through the reversible formation of an intramolecular disulfide bridge involving the cysteines present in the PAI subdomain, whereas the RED subdomain appears quite insensitive to redox potential. Circular dichroism experiments indicate that only the reduced form of the Prd domain is able to undergo the proper conformational change necessary for sequence-specific DNA binding. Nuclear extracts from different cell lines contain an activity that is able to reduce the PAI subdomain and, therefore, to control the DNA binding activity of this protein. Immunodepletion of nuclear extracts demonstrate that the protein Ref-1 contributes to the redox regulation of the Prd DNA binding activity. Given the modular nature of the Prd domain and the independent DNA binding specificity of the PAI and RED subdomains, we propose that this control mechanism should be involved in “switching” among different DNA sequences and therefore different target genes.

The paired box containing (Pax) genes encode for morphogenic transcription factors and constitute a multigene family conserved from nematodes to vertebrates (1–4). In mammals, the Pax gene family is composed of nine members (2, 4) grouped in four different classes according to the structural similarities present in the protein products (5). Pax proteins are defined by the presence of the paired domain, a 128-amino acid-long evolutionary conserved DNA-binding region (6, 7). The spatiotemporally restricted expression pattern of these proteins controls a variety of developmental decisions and, in adult life, contributes to the maintenance of differentiation state of several cell types (8–12). Some developmental functions of Pax proteins are conserved through evolution; for instance, the human PAX-6 is a master control gene for eye development in Drosophila, just as its fly eyeless homologous (13). Mutations of Pax genes give rise to abnormal phenotypes in mice and corresponding diseases in humans (5). Moreover, structural abnormalities or mis-expression may play a role in the generation or progression of human tumors (14–18).

The function of Pax proteins as transcriptional regulators depends on the intact function of the paired domain (7, 19). This region functionally consists of two distinct subdomains and recognizes specific DNA sequences (20, 21). The resolution of the crystal structure of the Prd domain of the Drosophila paired protein bound to a target DNA sequence confirmed the presence of two structurally distinct and independent subdomains (named PAI and RED, see Ref. 22), both containing a helix-turn-helix motif joined by a linker region (23). The presence of these distinct structural regions allows the Prd domain to recognize DNA sequences by using different binding modes. The Prd domain can bind as a monomer when it uses only the PAI subdomain or as a cooperation between the two subdomains (22). Alternative splicing may inactivate the binding function of the PAI domain; in this case, DNA sequences are recognized only through the RED subdomain, with oligomers of the protein assembled on a single DNA molecule (21, 24). Because of these different binding modes, Prd domains can recognize seemingly divergent sequences (20, 22, 23, 25).

The biological effects of Pax genes are linearly related to the amounts of the functional protein products. For example, both in humans and in mice, abnormalities due to PAX-6 mutations are semidominant; whereas the eye phenotype is more severe in the homozygous state, several abnormalities are evident also in the heterozygous state (26). Moreover, increasing the Pax-6 gene dosage in mice elicits also severe eye abnormalities (27). These observations suggest that functional levels of Pax pro-

* This work was supported in part by grants from MURST, AIRC, and “Fondi Interdiapartimentali Università di Udine” and by Target Project on Biotechnology (CNR, Rome, Italy, to C. F. and G. D.) and by “Progetto Strategico Biologia Strutturale” (CNR, Rome, Italy, to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: Prd, the paired domain of Pax proteins; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; ROS, reactive oxygen species; PCR, polymerase chain reaction; MALDI, matrix-assisted laser desorption ionization; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MS, mass spectrometry; TSH, thyrotropin; TFE, 2,2,2-trifluoroethanol.
teins are so strictly poised that even minor changes in the equilibrium state may give rise to a severe perturbation of the biological system. In turn, the functional activity of Pax proteins could be a sensitive switch to regulate cell function. Such a view predicts that either expression or molecular functions of Pax proteins could be the target of regulatory events. In the B cell lineage, for example, the transition from mature B cell to plasma cell is associated to a great reduction of Pax-5 gene expression (28). Moreover, thyrotropin (TSH), the chief regulator of the follicular thyroid cell functions, increases Pax-8 steady-state mRNA and protein levels in several follicular thyroid cell models (see also Ref. 29). In the past few years it became evident that oxygen free radicals and their by-products, collectively referred as reactive oxygen species (ROS), may influence gene expression (30). Also the activity of several transcription factors is controlled by ROS; the nuclear factor kappa-B (NF-kB) and the activator protein-1 (AP-1) are clear examples of this regulation (31). In the first case the ROS effects appear to be mediated by reduction/oxidation of a cysteine residue, located in the highly conserved Rel homology domain, that allows DNA binding only in the reduced state (32). The reduction/oxidation state of a single conserved cysteine regulates also the AP-1 DNA binding activity (33). More recently, it has been demonstrated that the redox potential controls the Pax-8-induced transcriptional activation of the thyroglobulin promoter (34), suggesting that the DNA binding activity of the paired domain could also be subjected to redox regulation. The aim of the present work was to test whether the redox potential can regulate the DNA binding function of the Prd domain. Our data demonstrate that the paired domain of Pax-8 and Pax-5 can recognize several DNA sequences only following a treatment with reducing agents. This effect occurs through the reduction of intramolecular disulfide bridges. The DNA binding function of the PAI subdomain is extremely sensitive to redox regulation, whereas the activity of the RED subdomain is unaffected. Therefore, the redox regulation may switch the DNA binding modes of Pax proteins. In addition we observed that nuclear extracts of several cell lines contain a temperature-sensitive activity able to mimic the effects of reducing agents.

EXPERIMENTAL PROCEDURES

Oligodeoxynucleotide Synthesis and Purification—Oligodeoxynucleotides were synthesized with an automated Applied Biosystem DNA synthesizer, model 380B, according to standard procedures and purified by fast performance liquid chromatography using a Mono-Q column (Amersham Pharmacia Biotech) eluted with an ammonium bicarbonate buffer containing 20 mM Tris-HCl, pH 7.6, 75 mM KCl, 0.25 mg/ml chicken egg white lysozyme (Sigma) (final concentration of 100 μg/ml) for 1 h at 30 °C and then frozen at −20 °C. After thawing, purified inclusion bodies were prepared by washing as described previously (36).

Bacteria expressing Pax-5 protein, after induction, were pelleted and resuspended in 10 ml of 5× TE (1× TE, 10 mM Tris, 1 mM EDTA, pH 7.5) for each gram of bacterial pellet. The bacteria were incubated with chicken egg white lysozyme (Sigma) (final concentration of 100 μg/ml) to an 8 μl area, 5 mM DTT, and then refolding of the protein was made by dialyzing twice the urea-solubilized protein against 10 volumes of TE containing 0.4 mg/ml arginine and twice 10 volumes of 10 mM phosphate buffer, 150 mM NaCl, pH 8.0, at 4 °C and centrifuged for 10 min at 32,000 × g. Then the protein was subjected to SDS-PAGE analysis determining a purification level of 70%.

EMSA Analysis—Double-stranded oligodeoxynucleotides, labeled at the 5′ end with 32P, were used as probes in gel retardation assays. The C site is a 24-mer whose upper strand is 5′-CATCTGCCCAGTCAGGTGTTTCCTGA-3′. The BS2 site is a 18-mer whose upper strand is 5′-CGAGGAGCTGGGGTCTGCTG-3′. The BS2 site is an 18-mer whose upper strand is 5′-TCTCAGGCGGCGGTGAGGTTCCG-3′. The BS2 site is an 18-mer whose upper strand is 5′-TCTCAGGCGGCGGTGAGGTTCCG-3′. The BS2 site is an 18-mer whose upper strand is 5′-TCTCAGGCGGCGGTGAGGTTCCG-3′.

The gel retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM Tris-Cl, pH 7.6, 75 mM KCl, 0.25 mg/ml bovine serum albumin with or without calf thymus DNA (50 μg/ml), 10% glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native polyacrylamide gel run in 0.5× TBE for 1.5 h at 4 °C. The gel was dried and then exposed to an x-ray film at −80 °C.

Mass Spectrometric Analysis—Samples used for binding experiments were alkylated by adding an equal volume of a 2.2 M iodoacetamide solution dissolved in the binding buffer and leaving the reaction mixture for 1 min in the dark at room temperature. Pax samples were removed from the excess of the blocking reagent by reversed phase high pressure liquid chromatography using a Vydac C4 column (250×4.6 mm, 5 μm) (The Separation Group) eluted with a linear gradient from 5 to 50% of acetonitrile in 0.1% trifluoroacetic acid, over 60 min.

Trypsin digestion was performed both on the purified carboxyterminal protein and on the native protein (100 μl of a 100 mM NH4HCO3 (100 μl), pH 7.0, at 37 °C overnight (enzyme-substrate, 1:50, w/w). The peptide mixtures obtained were lyophilized before analysis.

Protein samples were submitted to electrospray mass spectrometric analysis as described previously (38). Matrix-assisted laser desorption
ionization (MALDI) mass spectra were recorded using a Voyager DE MALDI-TOF mass spectrometer (Perspective Biosystems); a mixture of analyte solution, α-cyano-4-hydroxycinnamic acid, and bovine insulin was applied to the sample plate and dried in vacuo. Mass calibration was performed using the molecular ions from the bovine insulin at 2212.61 Da, as its standard. Temperature was controlled by a Haake F3 water bath. UV circular dichroism spectra are presented in terms of mean residue molar ellipticity (θ) (deg cm² dmol⁻¹), based on a mean residue weight of 110.4. The reported results are the smoothed average over 10 measurements.

Circular Dichroism—The purified Pax-8 Prd domain was used for CD spectroscopy. A Jasco J-600 CD/ORD spectropolarimeter interfaced to an Olidata computer for data collecting was used for measurements. Standard conditions were 75 mM KCl, 10 mM Na₂HPO₄, pH 7.4, 10 °C, using a 1-cm path length cuvette. Temperature was controlled by a Haake F3 water bath. UV circular dichroism spectra are presented in terms of mean residue molar ellipticity (θ) (deg cm² dmol⁻¹), based on a mean residue weight of 110.4. The reported results are the smoothed average over 10 measurements.

Cell Lines and Preparation of Nuclear Extracts—FRTL-5 cells were grown as described previously (39). NALM-6 cells were grown in RPMI medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. Cell nuclear extracts were prepared as described previously (40). Briefly, 10⁷ cells were washed once with PBS and resuspended in 1 ml of hypotonic lysis buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM PMSF, pH 7.9). After 10 min, cells were homogenized by 10 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation for 5 min at 500 × g at 4 °C in a microcentrifuge. Then, the nuclear proteins were extracted with 100 μl of buffer B (10 mM Hepes, 10 mM KCl, 0.1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM PMSF, pH 7.9). After incubating for 20 min at 4 °C, samples were centrifuged at 12,000 × g for 15 min. The nuclear extracts were then quantitated for protein levels according to the Bradford method (41) and used immediately for EMSA analysis or kept at -80 °C.

Western Blot Analysis—Extracts of FRTL-5 cells, prepared as described previously (40), were boiled in Laemmli sample buffer and resolved on a 12% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis onto nitrocellulose membrane (MSSI, Westboro, MA), Ref-1 was detected by a rabbit polyclonal-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Redox Potential Controls the DNA Binding Activity of Pax-5 and Pax-8 Transcription Factors—The Prd domain of Pax-5 and Pax-8 transcription factors contains three cysteine residues located at positions 37 and 49 (FAR domain) and 109 (RED domain). These residues are highly conserved in the whole family of Pax proteins. Protein samples were obtained by the expression in Escherichia coli of the Prd domain of Pax-8 and of the entire Pax-5. The Prd domain of Pax-5 was purified to homogeneity as previously reported (23) with minor modifications, and its structure was confirmed by mass spectrometry. In fact electrospray mass spectrometric analysis of this product revealed the presence of a single component presenting a molecular mass of 18,775.2 ± 1.1 Da (theoretical value, 18774.9 Da). Pax-5 was partially purified from inclusion bodies preparation (see “Experimental Procedures”). In order to identify whether Pax-5 and Pax-8 polypeptides are sensitive to redox potential in the interaction with their respective DNA-binding sites, the recombinant proteins were treated with fixed amounts of the reducing agent dithiothreitol (DTT) with the oxidizing agent diamine di-carboxylic acid bis[N,N-dimethylam- ido] (diamide) or were exposed to prolonged air conditions corresponding to an oxidative environment. The binding activity of the Pax-8 Prd domain to the oligonucleotide C (42) was examined by using electrophoretic mobility shift assay (EMSA). As shown in Fig. 1, panel A (lane 2), the binding of the Pax-8 Prd domain, following prolonged air exposure oxidation, was greatly reduced when compared with that obtained with the same protein maintained under reducing conditions (Fig. 1, panel A, lane 3). Accordingly, a treatment with diamide abolished the interaction of the Prd domain with the oligonucleotide C (Fig. 1, panel A, lane 4); however, subsequent addition to this sample of a DTT excess completely restored its binding capability (Fig. 1, panel A, lane 5), demonstrating that the effects elicited by oxidation are fully reversible. Similar results were obtained when the entire Pax-5 protein was assayed with the oligonucleotide H2A2.2 (43) (Fig. 1, panel B), thus suggesting that the process is context-independent and implying a general mechanism for all Pax proteins.

Next we investigated whether the DNA binding inhibition could be related to the dimerization/oligomerization state of the proteins; we performed SDS-PAGE analysis of the samples used in the EMSA experiments described above. The monomeric state of the Pax-8 Prd domain (Fig. 1, panel C) and of the entire Pax-5 protein (data not shown) was dependent on redox conditions. Both polypeptides were totally present as a single monomeric form only under reducing conditions (5 mM DTT, Fig. 1, panel C, lane 1, and data not shown). On the contrary, following a treatment with 5 mM diamide (Fig. 1 panels C, lane 2) or, albeit in lower amount, after prolonged air exposure (Fig. 1, panel C, lane 3), dimeric and oligomeric species were always present. The occurrence under oxidizing conditions of a faster migrating monomeric form (see the arrow in Fig. 1, panel C) can be interpreted as depending upon the presence of an intramolecular disulfide-bridged species with increased compactness (see below). The dimeric and oligomeric species were readily converted into the only monomeric form observed under reducing conditions by simple addition of a DTT excess to the oxidized samples (Fig. 1, panel C, lane 4), confirming the reversibility of the phenomenon and thus underlining a possible regulatory role for the oxidation process. It has to be understood that the DNA binding activity inhibition observed was not dependent on the dimerization/oligomerization process described so far since a consistent amount of monomer was still present in the oxidizing environment (Fig. 1, panel C, lanes 2 and 3).

Analysis of Redox State of the Cysteine Residues Present in the Pax-8 Prd Domain—In order to identify the cysteine residues involved in the redox regulation of the Prd domain of Pax-8, recombinant protein samples exposed to air or subjected to the reductive conditions used for DNA binding activity were alkylated with iodoacetamide. Following the reaction, the samples were desalted by reversed phase high pressure liquid chromatography yielding a single peak (data not shown), in agreement with the prominent monomeric form observed by SDS-PAGE analysis (Fig. 1, panel C). After tryptic digestion, the purified material was digested with trypsin, and the peptide mixtures obtained were directly analyzed by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Fig. 2 shows the mass spectra obtained for the digests of an air-exposed Prd domain sample of Pax-8 alkylated under native conditions (panel A), of an air-exposed sample of Pax-8 Prd domain (panel B), and of a reduced and alkylated Prd domain sample of Pax-8 (panel C), respectively. In all cases MALDI-MS analysis allowed the verification of most of the protein primary structure and the determination of the redox state of the cysteine residues. The nature of the peptides containing a disulfide bridge was confirmed by reduction of the peptide mixtures with dithiothreitol followed by MALDI-MS identification of the two reduced fragments (data not shown).

In the case of the air-exposed Prd domain sample of Pax-8 alkylated under native conditions (panel A), in addition to the signals at m/z 1836.7 and 2147.1, originated from peptides-(26–41) and -(103–122) carboxamidomethylated at Cys-37 and Cys-109, respectively, two clear peaks at m/z 2593.8 and 2904.2 were present in the spectrum. These signals were assigned to the peptide pair (26–41) + (45–52) linked by a disulfide bridge between Cys-37 and Cys-49 and to the peptide pair.
linked by a disulfide bridge between Cys-49 and Cys-109, respectively. Signals corresponding to other possible carboxamidomethylated or disulfide-bridged peptides were not present in the spectrum.

Similarly, in the case of the air-exposed Prd domain sample of Pax-8 (panel B) the two signals corresponding to the peptides containing a disulfide bridge were still present in the spectrum at \( m/z \) 2594.2 and 2903.9, respectively. Furthermore, MALDI-MS analysis revealed peaks at \( m/z \) 1779.8 and 2090.1 associated to peptides-(26–41) and -(103–122) containing Cys-37 and Cys-109 in reduced form; the molecular ions of the peptides-(26–41) carboxamidomethylated and -(103–122) carboxamidomethylated were not present in the spectrum.

On the contrary, the spectrum of the reduced and alkylated Prd domain sample of Pax-8 did not show the signals corresponding to the peptides containing the disulfide bridge (panel C).
C. The signals at m/z 873.9, 1836.9, and 2147.3 clearly indicated that peptides-(26–41), -(45–52), and -(103–122) presented fully carboxamidomethylated Cys-37, Cys-49, and Cys-109.

These results demonstrated that the native, air-exposed, and poorly functional Prd domain sample of Pax-8 was mainly constituted of two molecular species both having an intramolecular disulfide bridge and a free cysteine residue. The first component presented Cys-109 in a reduced form and a S–S bond between Cys-37 and Cys-49; the second one was characterized by the presence of a reduced Cys-37 and the disulfide bridge between Cys-49 and Cys-109. On the contrary, in conditions in which an effective DNA binding was detected, all the cysteine residues of the protein were present in a complete and reduced form.

The Oxidative Environment Affects the DNA Binding Activity of the PAI Subdomain—The Prd domain contains two subdomains (PAI and RED) with distinct and independent DNA binding activity (23). The N-terminal PAI subdomain (residues 1–72) is extremely conserved through the whole family of Pax proteins; it contains two cysteine residues at positions 37 and 49 present in each of the nine genes cloned up to now (23). The RED subdomain, at the C terminus (residues 77–128), is significantly less conserved than the other. However, the RED subdomain contains a cysteine residue at position 109 present in the whole family of Pax genes with the exception of the gsbn prd (23).

MALDI-MS experiments demonstrated that Cys-49 is the most reactive cysteine residue of the Prd domain of Pax-8 establishing, in the oxidized state, a disulfide bridge with either Cys-37 and/or Cys-109. Since Cys-49 contacts DNA (23), the DNA binding activity of the PAI subdomain should be extremely sensitive to redox conditions, whereas the DNA binding activity of the RED subdomain should be poorly sensitive to redox control. To test these predictions, we studied the DNA binding activity of both isolated PAI and RED subdomains under either oxidative or reductive conditions.

The PAI and RED subdomains were overexpressed as recombinant proteins in E. coli and purified to homogeneity (see conditions in which an effective DNA binding was detected, all the cysteine residues of the protein were present in a complete and reduced form.

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The binding activity of the isolated PAI subdomain was assayed with the oligonucleotide 11 that is recognized with high affinity. As shown in Fig. 3, the DNA binding activity of the PAI subdomain was completely abolished under oxidized conditions (lanes 2 and 3) and reversibly recovered following the addition of 5 mM DTT (lanes 4 and 5). The isolated RED subdomain was still able to recognize the C sequence of the Tg promoter, although with a lower affinity compared with the full-length construct. However, contrary to the results obtained with the PAI subdomain, the DNA binding activity of the RED subdomain was independent of the reducing environment (lanes 7–10).

The Oxidized State Abolishes DNA Sequence Discrimination of the Prd Domain—In order to better understand the reasons why the oxidized form of Pax-8 Prd domain was poorly able to recognize the C sequence, the DNA binding activity of the PAI subdomain was completely abolished under oxidized conditions (lanes 2 and 3) and reversibly recovered following the addition of 5 mM DTT (lanes 4 and 5). The isolated RED subdomain was still able to recognize the C sequence of the Tg promoter, although with a lower affinity compared with the full-length construct. However, contrary to the results obtained with the PAI subdomain, the DNA binding activity of the RED subdomain was independent of the reducing environment (lanes 7–10).

The Cys-37 → Ser Mutation Abolishes Redox Sensitivity of the Paired Domain DNA Binding Activity—In order to demonstrate that the Cys-37 to Cys-49 disulfide bridge specifically controls the DNA binding activity of the Prd domain, a Pax-8 Prd domain mutant was constructed, in which a Ser residue replaces the Cys at position 37. Since Cys-37 does not contact DNA (23), this mutation allow the interaction with DNA but, according to our model, it should abolish the sensitivity of the Prd domain to the redox conditions. The DNA binding properties of the C37S mutant are shown in Fig. 5. In contrast to the wild type protein, the mutant C37S is able to efficiently interact with the C sequence also in the oxidized state (lane 4).
Redox Regulation of Paired Domain DNA Binding Activity

The Cys-37 → Ser mutation abolishes redox sensitivity of the paired domain DNA binding activity. EMSA of the oxidized and reduced forms of the wild type Pax-8 Prd domain and of the Cys-37 → Ser mutant (C37S) of Pax-8 Prd domain incubated with the C sequence. The oxidized forms of the recombinant wild type Prd domain of Pax-8 or the C37S mutant were obtained by prolonged air exposure (lanes 2 and 4, respectively). The reduced forms were obtained by treatment with 5 mM DTT for 5 min at room temperature (lanes 3 and 5). At the end of the treatments, 45 ng of the wild type purified Pax-8 Prd domain or the C37S mutant samples were incubated with the oligonucleotide C (0.1 pmol) for 20 min at room temperature and loaded onto a native polyacrylamide gel for EMSA analysis. The arrow indicates the position of the protein-DNA complex.

These results greatly support the importance of the Cys-37 to Cys-49 disulfide bridge for redox regulation of Pax proteins. Since Pax-8 null mutant mice show developmental defects of the thyroid (5), the C37S mutant may provide an allele to investigate the role that redox mechanisms may play for Pax-8 function in the context of the whole animal.

A Reducing Environment Allows the Occurrence of the “Induced Fit” during the Formation of the Prd Domain-DNA Complex—Circular dichroism (CD) and nuclear magnetic resonance (NMR) structural analysis of the Pax-6 Prd domain revealed that this polypeptide is largely structureless in solution (44). However, upon binding to the recognition sequence, the protein undergoes a marked increase in α-helical content; this emphasizes the importance of a structural transition, occurring during recognition of the target DNA sequence, from a disordered to an ordered conformation typical of the induced fit model (45). To investigate whether the redox conditions were important in regulating the conformational state of Prd domain, the relationships between the redox state of cysteine residues and the conformation of the Prd domain of Pax-8 as judged from CD spectroscopy were investigated. When the Pax-8 Prd domain was analyzed, a spectrum with relative minima at 208 and 222 nm was observed (Fig. 6, panel A, filled triangles). The α-helix content, estimated from the mean residue ellipticity at 222 nm, was 15% under oxidizing conditions. Addition of a reducing agent to the oxidized sample (final concentration 0.1 mM DTT) induced a modification of the spectrum reflecting a small induction of α-helical content (19%) (Fig. 6, panel A, open circles).

The dehydrating solvent 2,2,2-trifluoroethanol (TFE) is known to generate structured states of peptides and in particular to stabilize helical regions (46). TFE is believed to induce α-helical structures in polypeptide chains which have intrinsic propensity for α-helix formation (47, 48). Therefore, spectra of Pax-8 Prd domain were collected in the presence of TFE. The addition of this solvent to the oxidized sample altered the protein structure; in fact, increasing the concentration of TFE led to a decrease in [θ] values at 208 and 222 nm, which is indicative for an increase of α-helical content. The amount of induced secondary structure reached a plateau (not shown) at 50% TFE (ν/v) resulting in 20% of α-helical content (Fig. 6, panel B, filled diamonds) for the oxidized form. However, in the presence of TFE and a reducing agent (0.1 mM DTT), the helical content reached a value of 46% (Fig. 6, panel B, open circles), thus providing evidence that a reducing environment is necessary for the full exploitation of the α-helical potential of the Prd domain of Pax-8. Therefore, the redox state controls the structure of the Prd domain by preventing (oxidized form) or by allowing (reduced form) a conformational change, under suitable conditions. The oxidized form could mimic a “closed” structural conformation that is unable to undergo a conformational change toward a more structured state. On the other hand, the reduced form could be considered as an “open” structure able to undergo a structural transition toward a more structured state of the protein.

To test whether these observations have a relevance in terms of DNA binding, spectra were also collected in the presence of the specific target DNA sequence (oligonucleotide C). While in the absence of reducing agents the spectrum of Prd domain after protein-DNA complex formation (Fig. 6, panel C, filled squares) was superimposable to the spectrum of the uncomplexed species; the addition of DTT (final concentration 0.1 mM) allowed the protein to undergo a clear conformational change with an increase of the α-helical content involving 33% of the residues (Fig. 6, panel C, open triangles). These results demonstrate that a reduced state is necessary for the occurrence of the hypothesis of the induced fit model proposed for Pax proteins (44). The lack of structural modifications of the oxidized form of the Prd domain was not due to the absence of DNA-protein complex formation because the experiments were performed in the absence of calf thymus DNA as competitor and in a molar excess of the specific oligonucleotide sequence C (r = 0.7). Under these conditions, in fact, a protein-DNA complex was clearly detected (see Fig. 4, lane 2 and data not shown).

A Nuclear Factor Is Able to Increase the DNA Binding Activity of Prd Domain—To result in an efficient DNA binding activity, recombinant Jun and Fos-Jun complexes (i.e. AP-1) require the presence of reducing agents such as DTT or, alternatively, an additional protein present in nuclear extracts (31). This protein, subsequently identified as Ref-1 (49), is able to stimulate the DNA binding activity of recombinant Fos and Jun, NF-κB, Myb, and other members of the ATF/CREB family in a redox-dependent manner. The mechanism involves a conserved cysteine basic amino acid motif (KCR) that is present in all Fos- and Jun-related proteins identified to date (49).

To test whether factor(s) present in nuclear extracts from cells constitutively expressing Pax-8 (FRTL-5) contribute to the redox regulation of DNA binding, the purified Prd domain of Pax-8 protein was assayed for DNA binding activity in the presence or in the absence of nuclear extracts. The Prd domain of Pax-8 exhibited a high degree of DNA binding activity only in the presence of nuclear extracts (Fig. 7, lane 5), suggesting that nuclear extracts contain a factor(s) that increases the DNA binding activity of the Pax-8 transcription factor. This effect is not due to the simple presence of proteinaceous stabilizing agents, because the simple addition of an excess of serum albumin was not able per se to increase the binding activity of the recombinant protein (Fig. 7, lane 2). The nuclear activity was sensitive to heat treatment (Fig. 7, lane 6), suggesting that
the activity is of a polypeptide-like nature. Moreover, this activity was abolished by treatment of the nuclear extracts with oxidizing agents (diamide) (data not shown), suggesting that the nuclear factor(s) contains thiol groups essential to promote the DNA binding activity of Pax-8. The same results were obtained with the entire Pax-5 recombinant protein by using nuclear extracts from NALM-6 cells (data not shown) suggesting a general mechanism for Pax proteins.

One possibility is that the nuclear protein Ref-1 was involved in the increase of the DNA binding activity of Pax transcription.

**FIG. 6.** UV circular dichroism spectra of the Pax-8 Prd domain in oxidizing (Ox) and reducing (Red) conditions. Panel A, CD spectra of the Prd domain of Pax-8 under oxidizing (prolonged air exposure) (filled triangles) and reducing conditions (0.1 mM DTT) (open circles). Panel B, CD spectra of the Prd domain of Pax-8 in the presence of TFE 50% v/v under oxidizing (prolonged air exposure) (filled diamonds) and reducing conditions (0.1 mM DTT) (open circles). Panel C, CD spectra of the Prd domain of Pax-8 in the presence of 0.4 μM oligonucleotide C under oxidizing (prolonged air exposure) (filled squares) and reducing conditions (0.1 mM DTT) (open triangles) after correction for the contribution of the oligonucleotide. The spectra were recorded at a protein concentration of 0.33 mM in 10 mM phosphate, 75 mM KCl, pH 7.4, at 10 °C and 1-cm path length. The helix content was estimated from the mean residue ellipticity at 222 nm, assuming that for a 100% helical peptide this value is -33,000 degrees cm² dmol⁻¹ at 0 °C, and at higher temperatures is attenuated 0.3% per °C before unfolding (65, 66) and is represented in the inset of each panel.
Redox Regulation of Paired Domain DNA Binding Activity

FIG. 7. DNA binding activity of Prd domain is stimulated by a nuclear factor. Panel A, DNA binding activity of recombinant Pax-8 Prd domain. Purified Pax-8 Prd domain was incubated at room temperature in the presence of 5 μg of bovine serum albumin (lane 2), 5 mM DTT (lane 3), 5 μg of crude FRTL-5 cell nuclear extract (lane 5) or 5 μg of heat-denatured FRTL-5 nuclear extract (kept at 90 °C for 5 min and then cooled before incubation with the probe DNA) (lane 6). The crude FRTL-5 nuclear extract without Pax-8 Prd domain is represented in lane 4. Then the proteins were assayed for binding to a 25-base pair 32P-labeled oligonucleotide C (0.1 pmol) following preincubation with 1 μg of calf thymus DNA for 5 min at room temperature. Protein-DNA complexes were resolved on a 10% native gel and visualized by autoradiography of the dried gel. The arrow indicates the position of the Prd-DNA complex. To test for the specificity of the complex, a competition assay with cold specific and nonspecific oligonucleotide was performed. As expected, the specific C oligonucleotide efficiently reduces the Prd-DNA complex (lanes 7 and 8), whereas the nonspecific BS2 oligonucleotide is almost ineffective (lanes 9 and 10). Signals at the top of the wells are due to precipitated material from nuclear extracts.

FIG. 8. Inhibition of the Prd domain DNA binding activity by immunodepletion of Ref-1 from FRTL-5 nuclear extracts. Panel A, FRTL-5 nuclear extracts (5 μg) (lanes 4–8) were incubated for 30 min at room temperature with 3 μl (lane 5) or 15 μl (lane 6) of anti-Ref-1 rabbit polyclonal antibody (Santa Cruz Biotechnology) and with 3 μl (lane 7) or 15 μl (lane 8) of total rabbit IgG (Sigma) linked to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) at a final concentration of 50 μg of antibody per ml of resin. Then 5 μl of each cleared supernatant was assayed for Pax-8 Prd domain redox activity by gel retardation assay using 90 ng of Prd domain (as described under “Experimental Procedures”). The Pax-8 Prd domain used was tested for its DNA binding activity with the oligonucleotide C under oxidizing (lane 2) and reducing (lane 3) environment and in the presence of crude FRTL-5 nuclear extract (lane 4). The arrow indicates the position of the protein-DNA complex. Panel B, the cleared supernatants were tested for the effective presence of Ref-1 protein in Western blot, using the anti-Ref-1 antibody (Santa Cruz Biotechnology). The numbers correspond to those in the description of panel A.

DISCUSSION

Pax genes are important regulators of developmental pathways, and in adult life, they hold a prominent role in the maintenance of differentiation state of several cell types (5). Because of the central role that these genes play in differentiation and development, it may be expected that Pax genes are subjected to several levels of regulation. It has been recently demonstrated that alternative splicing for some of these genes, i.e. Pax-6 (21), Pax-8 (24), Pax-3, and Pax-7 (51), could give rise to protein isoforms with quite different DNA binding specificities. Alternative splicing, if regulated, could represent a potential mechanism to switch among a subset of different target genes. Besides this pre-translational control, additional forms of regulation could be achieved by post-translational mechanisms, such as phosphorylation (52–54). In the last few years increasing evidence suggested that redox potential can be considered a useful mechanism able to modify the DNA binding activity of several Pax factors. This observation seems to be particularly interesting in the case of Pax-8 regulation. In fact, it has been recently demonstrated that TSH increases the expression levels of the Ref-1 protein (50); therefore, it could be hypothesized that the modulation of the Pax-8 DNA binding activity could be under the control of TSH through the regulation of Ref-1 expression.

Redox Regulation of Paired Domain DNA Binding Activity

<table>
<thead>
<tr>
<th>Competitor</th>
<th>C</th>
<th>BS2</th>
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<tr>
<td>Molar Excess</td>
<td>20</td>
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ing the PAI and RED subdomains (23) which are able, per se, to recognize different DNA sequences3 (see also Ref. 24). The PAI subdomain contains two conserved Cys residues, one of which, Cys-49, is believed to establish specific contacts with base pairs (23). The electrophoretic mobility shift assays, here described for Pax-5 and Pax-8 Prd domain, clearly demonstrated that binding to the corresponding DNA targets is extremely sensitive to redox conditions. MALDI-MS analysis of the Pax-8 construct showed that Cys-49, which can form a disulfide bridge with either Cys-37 or Cys-109, is highly oxidizable, thus accounting for the loss of DNA binding activity. Furthermore, EMSA experiments on the separated PAI and RED subdomains demonstrated that although the binding activity of the PAI subdomain was heavily subjected to redox potential control, the RED subdomain activity was unaffected by the oxidizing environment. This insensitivity could be due to two, not mutually exclusive, reasons as follows: (i) Cys-109 does not establish contacts with DNA and/or its redox state does not influence other DNA-contacting residues; (ii) the protein molecules, in which the Cys-37 to Cys-49 disulfide bridge occurs, still present the Cys-109 in the reduced state. 

MALDI-MS analysis did not allow the quantitative measurement of the relative abundance of the Cys-37 to Cys-49 or of the Cys-49 to Cys-109 S–S bridges in the samples. However, theoretical analysis indicates that the former disulfide bridge is more favored compared with the latter. In fact, the reactivity of a cysteine residue is enhanced by the neighboring basic amino acids through the formation of a thiol-anion base pair (58). Theoretical isoelectric local points calculation (pI) of the peptides surrounding cysteine residues in Prd domain predicts a very low reactivity for Cys-109 (local pI = 2.8), in comparison to either Cys-37 (local pI = 8.9) or Cys-49 (local pI = 8.9). The binding phenotype of the C37S mutant demonstrates the relevance of the Cys-37 to Cys-49 disulfide bridge controlling the Prd domain. Since the redox potential controls the binding activity of the PAI subdomain but not that of the RED subdomain, changes in the redox potential could act as a switch between a DNA binding mode in which the PAI subdomain is involved to a DNA binding mode based only on the activity of the RED subdomain. A similar molecular genetic “switch” has been demonstrated for the OxyR-controlled regulon of hydrogen peroxide-inducible genes in Salmonella typhimurium and E. coli (57). A conformational change of OxyR, upon direct oxidation of the protein induced by exposure of the cells to hydrogen peroxide, leads to unleash its transcriptional activating potential. This regulation goes through a reversible intrachain disulfide bond formation between two Cys residues (59). The conformational change allows OxyR to interact with the same promoters (oxyr, katG, and ahpC) in different manners. For example, the OxyR protein can function both as an activator (reduced form) or as a repressor (oxidized form) in regulating its own expression through the binding of the oxyr promoter (57).

CD experiments have established that the oxidized state of the cysteine residues in the Prd domain induced structural variations leading to an interference with the proper conformation useful for DNA binding. Thus, the redox state of these residues controls the Prd domain potential in undergoing the conformational switch necessary for sequence-specific DNA binding. Also for the tumor suppressor protein p53, redox changes could influence the biological activity by modulating its DNA binding activity through effects on protein conformation. More specifically, reduction of thiols favors the tertiary folding of p53 in a form permissive for DNA binding (60). Similar findings have been described for the oncprotein Myb (61); the redox state of a unique cysteine residue (Cys-43) could function as a molecular sensor for a redox regulatory turning on-off specific DNA binding. Here, we propose that the redox potential could not simply turn on-off the binding activity of Pax proteins, but it would control subsets of target genes subjected to the regulation of Pax gene products. In the reduced state, Pax proteins would regulate transcriptional units in which sequences recognized by the PAI subdomain are present; in the oxidized state the effect of Pax proteins would be directed to transcriptional units in which the RED subdomain is sufficient for binding.

The cells realize efficient production of ATP mainly through the glucose oxidation. However, this event leads to the generation of reactive oxygen species (ROS) such as H2O2, O2−, and ‘OH radicals, that are by-products in electron transport processes into the mitochondria. To protect themselves from the harmful effect of oxygen, cells use primarily two defense mechanisms, the enzymatic systems of catalase and superoxide dismutase, and the glutathione and TRX systems, which contribute to create an intracellular reducing environment. Therefore, through the induction of antioxidant systems, ROS constitute a useful tuning device for signal transduction. It is well established that the redox state affects cell growth and several differentiated functions by modulating signaling pathways activated by cell-surface receptors. This is the case of the cascades induced by cytokines such as tumor necrosis factor-a or interleukin-1β (31). Besides endogenous oxidative stress generated metabolically in multicellular organisms, cells are exposed to oxidative stresses originated from other tissues as well as from neighboring cells. For example, the lymphocytes are exposed to oxidative stress when they migrate in inflammatory regions where oxidants are generated by neutrophils and other phagocytic cells in the processes accompanying phagocytosis. This observation seems particularly interesting in the case of Pax-5, which is the only Pax gene that plays a primary role in lymphoid tissue. During maturation of B cell, this gene’s results are completely turned off when the mature B cell transforms to plasma cell (28).

Finally, a series of investigations have established that TSH is able to induce the production of H2O2 that is essential in the iodination and in the coupling reactions for the synthesis of thyroid hormones (62, 63). The finding that TSH increases the DNA binding activity of Pax-8 through a redox mechanism reinforces the hypothesis that ROS may serve as common intracellular signals in specific tissues (30, 64).

Acknowledgments—We thank Prof. Alfonso Colombatti, Prof. Roberto Di Lauro, Prof. Gennaro Marino, and Prof. Franco Quadrifoglio for helpful discussion and comments on the manuscript. We also thank Silvia Lolini for DNA synthesis, Carlo Lo Cascio for the artwork, and Dr. David Cimarosti and Dr. Alessandro Zecca for the help in recombinant protein expression and purification.

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Redox Potential Controls the Structure and DNA Binding Activity of the Paired Domain

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doi: 10.1074/jbc.273.39.25062

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